Journal of Surgical Oncology 62:194–200 (1996)

Antisense Oligonucleotide Intralesional Therapy for Human PC-3 Prostate Tumors Carried in Athymic Nude Mice

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Previously we reported hemorrhagic necrosis in human-derived PC-3 prostate tumors, in athymic nude mice, produced by the intralesional injection of antisense oligonucleotides (oligos) directed against mRNAs encoding transforming growth factor-α (TGF-α) and its target, the epidermal growth factor receptor (EGFR). We now describe our experience with these oligos in treating additional mice with various doses and modes of administration. During prolonged treatment, a dose-response effect was observed, with the optimal dosage consisting of the combination of 400 μg of each oligo. Although responses varied, based upon amount and how oligos were administered, we found that tumors were best treated when initially less than 156 mm^3. Intralesional inoculations produced necrosis and yielded responses, ranging from complete response (CR) or cure to partial responses (PR) in 9 of 12 tumors treated with full dose (400 μg of each oligo) and 1 of 1 treated with 800 μg of each oligo, against a large tumor. Included among the 9 positive responses with full-dose administration were 2 tumors that regressed (one completely). A single tumor treated with twice (2×) the normal dosage (800 μg of each oligo) also regressed. A single tumor treated with half (1/2) dose (200 μg of each) progressed similar to controls, as did 3 of 12 treated with the full dose. Limited experience with ALZET diffusion pumps gave CR (1 of 3) or PR (2 of 3) in 100% of tumors treated (including one mouse cured of multiple tumors in a five day period). It appears that multiple inoculations consisting of 400 μg of each oligo is most effective against these tumors, particularly when administered against tumors of <156 mm^3 in initial size.

KEY WORDS: **antisense oligonucleotides, growth factor deprivation therapy, prostate cancer**

INTRODUCTION

Antisense oligonucleotides are artificial sequences of nucleotide bases synthesized complementary to specific mRNA. When hybridized to specific mRNA, they can regulate cellular proliferation, programmed cell death (apoptosis), or other biologic activities. Because of their ability to regulate the expression of proteins acting as either growth factors, growth factor receptors, or those having suppressor or oncogenic activities, antisense oligos are increasingly being entered into clinical trials against diseases for which specific proteins have been targeted for suppression. None of these oligos, however, involves treatment of solid malignant tumors. Among the first antisense compounds entering clinical trials are an oligo directed against c-*myb* for treatment of chronic

Accepted for publication February 26, 1996.

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myelogenous leukemia [1] and one directed against p53 for treatment of acute myelogenous leukemia (AML) [2]. The ISIS Corporation (Carlsbad, CA) is currently performing clinical trials with oligos effective against condyloma acuminatum (ISIS 2105), cytomegalovirus (CMV) retinitis in acquired immunodeficiency syndrome (AIDS) patients (ISIS 2922), psoriasis, and ulcerative colitis (ISIS 2302) [2]. In addition, the Hybridon corporation (Worcester, MA) is in trials with GEM 91 for human immunodeficiency virus (HIV) infection [3].

In several previous reports, we have described the rationale for the development of oligos directed against mRNA encoding the autocrine loop consisting of transforming growth factor-α (TGF-α) and its binding site, the receptor for epidermal growth factor (EGFR). These oligos are effective against human derived prostate tumors both in vitro [4], where these oligos significantly inhibited growth of human derived PC-3 prostate cancer cells, and in vivo [5], producing hemorrhagic necrosis in PC-3 tumors carried in athymic nude mice [6]. These studies were conducted in an effort to demonstrate the feasibility of growth factor deprivation therapy for hormone-insensitive prostate tumors [7].

We now report our experience treating established tumors over a prolonged period of time, and also look at the effects of tumor size and inoculation dosage. Treatment was administered either by direct intralesional inoculation or by release from implanted ALZET diffusion pumps.

MATERIALS AND METHODS

PC-3 Tumor

The PC-3 tumor line was established in 1979 from a bone marrow metastasis of human prostate cancer [8]. These hormone-insensitive cells produce and secrete prostate-specific acid phosphatase (PAP), but not prostate-specific antigen (PSA). They also secrete and respond to TGF-α [9].

In Vitro PC-3 Cell Maintenance

Stock PC-3 (ATCC #CRL 1435) cells were maintained in Ham's F12K (Irvine) media supplemented with 7% fetal bovine serum (FBS) (Sigma; St. Louis, MO), at 37°C, in a 5% CO_2 incubator.

Establishment of In Vivo PC-3 Tumors

For most experiments, PC-3 tumors were established in athymic nude mice, using $1–3 \times 10^6$ in vitro propagated cells. Tumors appeared within 4 weeks when using either fresh aliquots of PC-3 cells or cells subcultured from explants. Tumors were also established on occasion, using excised tumor masses of approximately 0.1 cm^3 in size.

Antisense Oligonucleotides

Antisense oligos (39 mer) were synthesized (Operon; Alameda, CA) complementary to 18 bases located 5′ and 3′ from the AUG mRNA translation initiation codon. Gene sequences were obtained using the GenBank (Mountain View, CA) database. Antisense Oligo Probe #1, designated *MR-1* and directed against the mRNA encoding TGF-αa, consisted of

5′ C-T-G-T-C-C-A-G-C-C-G-A-G-G-G-G-A-C-C-A-
T-T-T-T-A-C-G-G-G-C-G-G-G-C-G-G-G-C-A 3′

Antisense Oligo Probe #2, designated *MR-2* and directed against the mRNA encoding EGFR, consisted of

5′ G-G-C-C-G-T-C-C-C-G-G-A-G-G-G-T-C-G-C-A-
T-C-G-C-T-G-C-T-C-C-C-C-G-A-A-G-A-G-C 3′

All oligos were phosphorothioated at each of three terminal bases at both the 5′ and 3′ ends to prevent exonuclease degradation [11].

Oligo Dosage and Tumor Treatment

Oligos were dissolved in 0.01 M Tris–HCl, at pH 7.4, containing 10 mM EDTA. For tumors removed for histologic evaluation, 200 μg of each oligo was used for 2–3 consecutive days. Tumors treated by intralesional inoculations received 0.1 ml of solutions containing either 200, 400, or 800 μg of each oligo; 400 μg of each oligo was then determined to be the "standard full dose" for intralesional injections. In experiments using an ALZET (Palo Alto, CA) diffusion pump, the equivalent of two doses (400 μg of each oligo), previously determined to produce hemorrhagic necrosis, was used in a pump designed for release over a 7-day period. In all experiments, tumors were periodically measured along 2–3 axis using vernier calipers and plotted in a semilogarithmic manner.

Controls

Controls both for nonspecific treatment activity and for human TGF-α and EGFR sequence specificity consisted of both RPMI or the buffered Tris oligo diluent injected directly into similar tumors carried in nude mice. Treatment of a histologically similar, rapid-growing, androgen-independent Dunning R3327 AT-3 rat prostate tumor [10], carried in Copenhagen X Fischer rats, was also employed. Absence of effect in either control demonstrated both the specificity of the antisense oligos to human TGF-α and EGFR and a lack of nonspecific toxicity.

Evaluation of Treatment

Complete responses (CR) were defined as a complete regression of treated tumors. Partial responses (PR) were defined as either reduction in tumor size, lack of tumor progression, or progression at a rate less than controls.

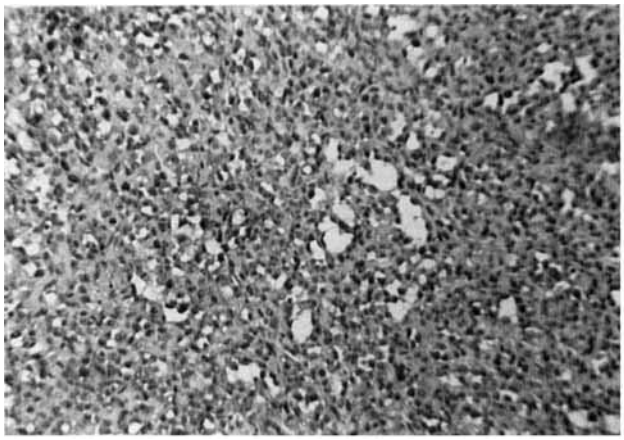

Fig. 1. Untreated PC-3 tumor. ×300.

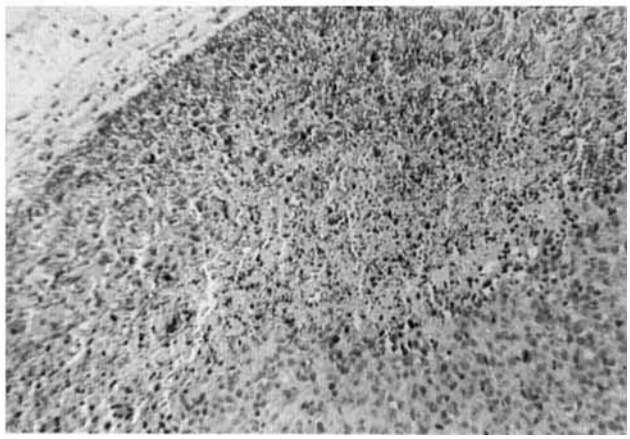

Fig. 2. PC-3 tumor treated with MR12. ×300.

Animal Care

Animals were anesthetized using Ketamine administered intraperitoneally in a 0.1-ml volume for all surgical procedures. Euthanasia was performed under methods consistent with the recommendation of the Panel of Euthanasia of the American Veterinary Medical Association.

RESULTS

Intratumoral Treatment

Nude mice bearing established solitary PC-3 tumors, 5–10 mm in diameter, were injected intralesionally daily with 200 μg of either MR1 (directed against mRNA encoding TGF-α), MR2 (directed against mRNA encoding EGFR), or the MR12 combination in a 0.1-ml volume for 2–3 consecutive days. Oligos were diluted from stock solutions with RPMI media. At 24–48 hr after the final injection, the tumors were removed for pathological evaluation. Compared to injected control tumors (Fig. 1), those PC-3 tumors treated with MR1 or MR2 oligos, either alone or in combination, showed hemorrhagic necrosis and immune cell infiltration (Fig. 2). The combination treatment was determined, in a blind evaluation, by a Board Certified Pathologist to be the most effective. Treatment of controls with RPMI media produced no nonspecific necrosis, nor was any produced while treating the similar, but murine-derived, Dunning AT-3 tumor with the MR12 oligo combination.

Prolonged Treatment

In order to measure effects on tumor size and progression, mice bearing PC-3 tumors were inoculated intralesionally with various amounts of the MR12 oligo combination over a period of time. In order to produce maximal effects on the treated tumors, while using a standard intralesional dose of oligos, various numbers and timing of inoculations were used.

In a series of six experiments (Table I), a total of 23 mice bearing solitary tumors, of at least 2-mm diameter, were paired off with others of comparable size for a series of treatment studies. A total of nine tumors were utilized as controls. Of the remaining 14 tumors, 12 received intralesional inoculations of a standard MR12 dose of oligos, consisting of 400 μg of each (MR1 and MR2) in a 10- to 20-μl volume; one received a half-dosage (200 μg of each); and one received a dosage 2× standard level (800 μg of each). Of the 12 treated with standard dosage, one complete response (CR) and eight partial responses (PR) were obtained (CR + PR = 75%). PR were further characterized as either a reduction in tumor size (1 of 9; full dose treated, and 1 of 1 in treatment of a large tumor with 2× dosage), or by progression at a rate less than that of controls (8 of 9; full dose treated). PR were distributed between each of the six experiments studied. We also report a lack of response (NR) in three instances in which tumors progressed at rates comparable to those of their controls. Two of these NR were in a single experiment, which also included one PR having a final tumor progression rate 74% below that of the control (Table I, experiment 3). A single tumor that was treated with half-dosage of oligos progressed at a rate identical to that of two controls.

The results of a single experiment that best represents our findings are shown in Figure 3. Five tumors are distributed between two control mice and three experimental animals, illustrating both the effectiveness and dose-response nature of this treatment. Effectiveness ranged from no response (NR) in the half-dose treated to a CR and one PR with the full standard dose.

We found that of those tumors that responded to standard-dose treatment, all but one were initially smaller than 170 mm^3. Two of the three tumors that failed therapy, initially measuring 156 and 254 mm^3, were among the largest tumors treated with full dosage; one of these mice

TABLE I. Antisense Treatment of Human PC-3 Prostate Tumors in Athymic Nude Mice*

Experiment	No. of tumors treated	Dose (each oligo) (μg)	Effect vs. control(s)	Notes
1	3	400	CR	Complete tumor regression
		400	PR	68% less tumor progression
		200	NR	Progressed comparable to controls
2	1	800	PR	40% reduction in tumor size
3	2	400	PR	58% less tumor progression
		400	PR	75% less tumor progression
4	3	400	PR	74% less tumor progression
		400	NR	Progressed comparable to control
		400	NR	Progressed comparable to control
5	2	400	PR	13% reduction in tumor size
		400	NR	Progressed comparable to controls
6	3	400	PR	43% less tumor progression
		400	PR	73% less tumor progression
		400	PR	70% less tumor progression

CR, complete response; PR, partial response; NR, no response.
*Note the reduction in tumor size that occurred in Experiments 1, 2, and 5.

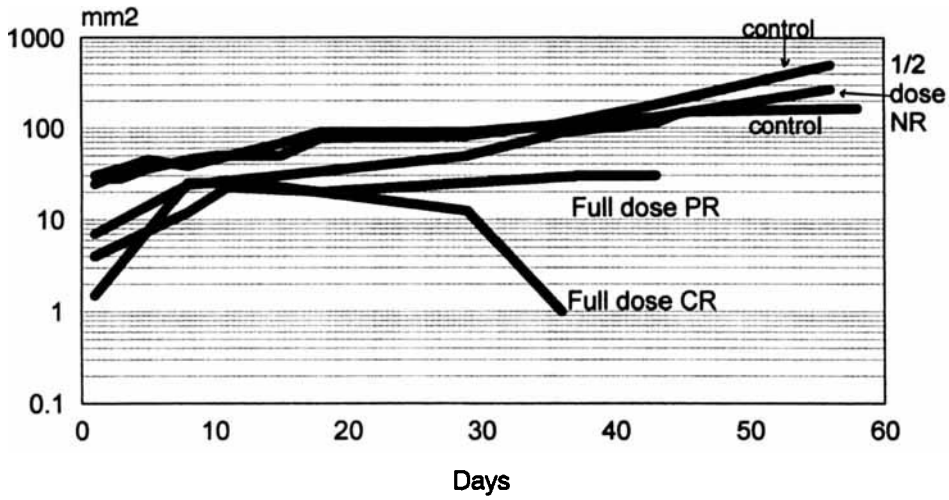

Fig. 3. Growth curve and survival of treated and untreated PC-3 tumors treated with full or half-dosage administrations of MR12.

developed a second tumor (midway in the treatment), which was then inoculated separately with an additional 8 full doses. One tumor that failed standard therapy, however, initially measured 40 mm^3. The largest treated tumor initially measured 270 mm^3. It was treated with four administrations of 2× dosage and responded favorably (PR) with a 40% reduction in tumor size compared to its paired, progressing, control.

Systemic Delivery

In an effort to administer this treatment systemically, the MR12 combination of both oligos via an ALZET diffusion pump was administered to three nude mice carrying PC-3 tumors (one of which carried multiple tumors). The pumps were inserted subcutaneously and contained 400 μg of each oligo in a 0.1-ml reservoir regulated to diffuse over a 7-day period. A CR was noted by the fifth day in the first mouse, which bore multiple tumors, the largest of which measured 0.3 × 0.8 cm. This mouse remained alive throughout the 7 days of oligo diffusion but died of infection the following week after surgery to remove the pump. This additional surgery was performed in an effort to determine the period of disease-free survival. Two additional mice, similarly treated, had demonstrated PR. One had a tumor that progressed at a rate slower than one untreated control; another had a tumor that remained either unchanged or slightly smaller in size, while its control progressed. This latter mouse had a

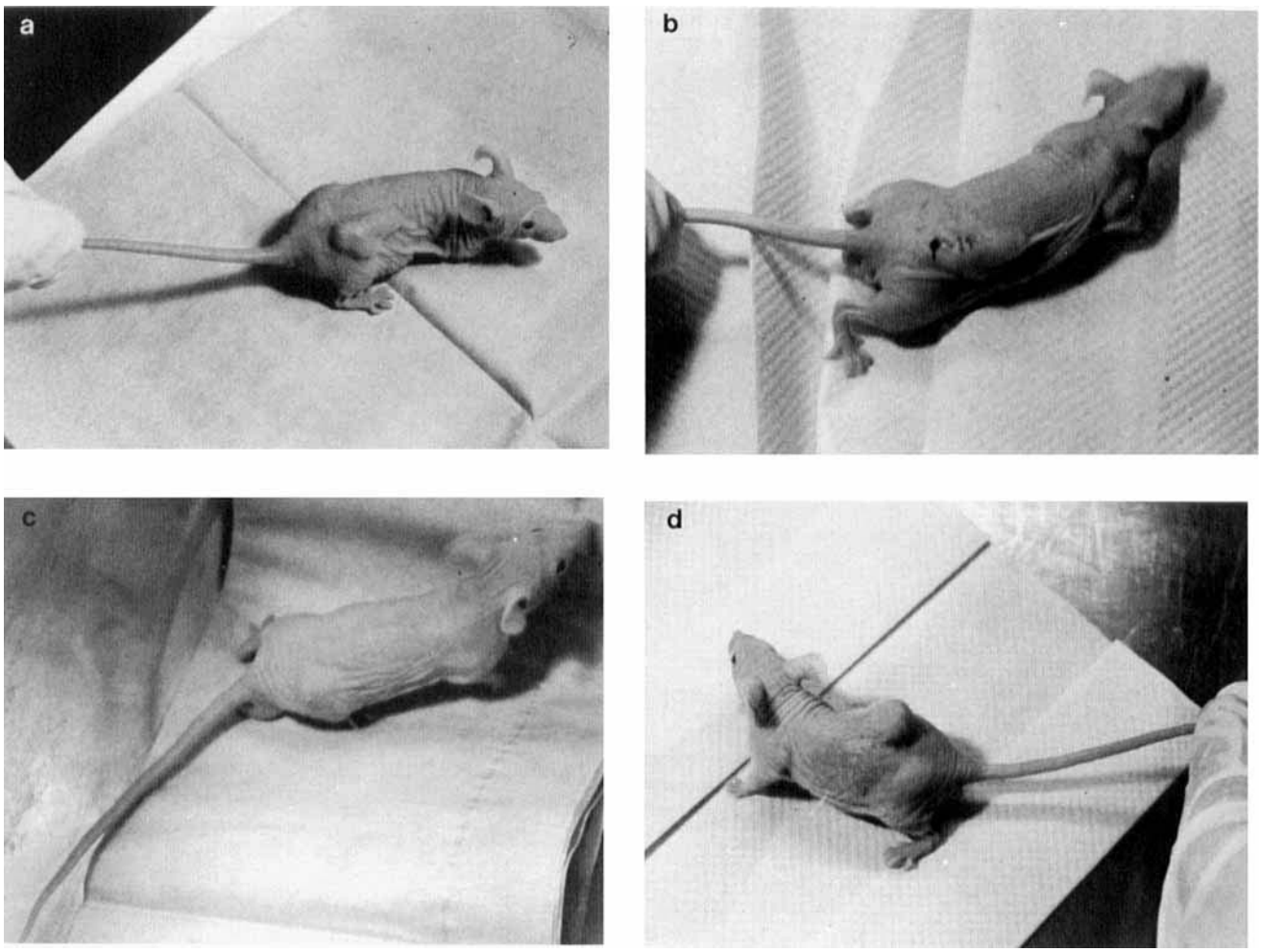

Fig. 4. **a:** PC-3 tumor adjacent to and beneath the ALZET diffusion pump at 1 week after insertion. **b:** The same mouse 1 week following insertion of a second pump. **c:** Control tumor similar in size to that treated in a. **d:** Progression of an untreated control tumor during the treatment period.

second pump inserted 14 days following the first, and the tumor remained small during this period as well. Figure 4a shows a nude mouse bearing a PC-3 tumor and ALZET diffusion pump. Figure 4b shows the same mouse one week later following insertion of a second pump. Figure 4c shows size of initially treated PC-3 tumor. Figure 4d shows progression of an untreated tumor.

DISCUSSION

The clinical efficacy of using antisense oligos for the treatment of human disease is currently being evaluated [1–3]. These compounds are specifically able to block protein expression through their regulation of both transcription and translation pathways. Selection of an appropriate mRNA target, whose encoded protein is critical to the pathogenic mechanism of disease, is therefore crucial. In a recent review, we described the process of choosing appropriate targets and proposed that in hormone insensitive tumors, of prostate or breast origin, antisense oligos could form the basis of a new form of therapy [7]. Growth factor deprivation therapy targets those proteins thought to regulate autocrine loops associated with cancer growth by inhibiting expression of either the growth factors themselves and/or their binding site receptors. These loops would be particularly important in growth regulation following the development of hormone insensitivity.

Specifically, we have evaluated the autocrine loop involving TGF-α and EGFR. This particular autocrine loop was selected because of its suspected role in prostate cancer progression and because of gene sequence homology, particularly with epidermal growth factor (EGF) [12], several additional factors implicated in tumor growth may be inhibited. PC-3 cells contain mRNA for TGF-α [13] and its binding site EGFR is implicated by its increased mRNA presence in prostate cancer compared to benign hyperplasia [14]. Lastly, EGFR is homologous

to the erb-b2 oncogene, which when either overexpressed or functionally altered is implicated in several forms of cancer [15]. Hence utilizing two oligos, we simultaneously disrupt numerous regulatory pathways implicated in tumor progression and oncogenesis in prostate tissue. We used a combination of oligos because in our in vitro studies [4,7] we found it to produce a greater amount of proliferative inhibition. Also, in our in vivo studies [5,6], the combination was judged more potent in a blind evaluation. The combination of multiple agents is common in chemotherapeutic protocols, and in this instance using two agents to inactivate an autocrine loop would probably be not only more effective, but also less likely to lead to resistance. In this instance, the use of two agents to inactivate this autocrine loop was selected because of its suspected role in prostate cancer progression and because of gene sequence homology, particularly with epidermal growth factor (EGF) [12], several additional factors implicated in tumor growth may be inhibited. PC-3 cells contain mRNA for TGF-α [13] and its binding site the EGFR is implicated by its increased mRNA presence in prostate cancer compared to benign hyperplasia [14]. Lastly, since EGFR is homologous to the erb-b2 oncogene, which when either overexpressed or structurally altered, is implicated in several forms of cancer [15], oligos complementary to EGFR may also limit expression of this oncogene. Hence using two oligos, we disrupt numerous regulatory pathways simultaneously within a single autocrine loop that has been implicated in tumor progression and oncogenesis.

Although direct inoculation of tumors was effective, systemic administration of oligos using diffusion pumps appears to have a better response rate (3 of 3, with 1 CR). Furthermore, this may be further optimized using both greater amounts of oligos, and sequential implantation of replacement pumps.

During these experiments, we identified several problems that included (1) difficulty establishing tumors of reproducible size within a predictable time frame; (2) the exclusion of many mice that developed either multiple tumors, solid tumors distal from the inoculated area, or spontaneous skin lesions; and (3) animals that developed fluid containing cysts, which upon treatment drained and disappeared. In addition, nude mice are extremely susceptible to infections that preclude removal of inserted pumps for the study of disease-free intervals. All are common problems with athymic mice. In order to remedy these difficulties, we experimented with PC-3 cells prepared by different methods for implantation. These methods included in vitro-grown cells obtained from either fresh stock cultures or recent tumor explants. We also tried surgically to implant small tumor pieces themselves. We found that the fastest and most consistently growing tumors were obtained from inoculation of single cell suspensions of PC-3 cells outgrown from either fresh ATCC stocks or recently explanted tumors. Tumor lines that were propagated for a period of time appeared increasingly to lose their ability to produce a high percentage of takes and developed more slowly. However, once tumors developed, we could identify no differences in their response to treatment.

In conclusion, we have described our experience with antisense oligos for the in vivo treatment of human prostate cancer within a nude mouse model. These findings add to those previously reported that demonstrate the biological activity of these antisense oligos against hormone-insensitive prostate tumors in vitro [4], in vivo [5,6], and also within the apoptotic pathway [16] where antisense oligos directed against EGFR enhanced the expression of the bcl-2 protein in a manner similar to that observed following androgen deprivation therapy. Delivery mechanisms of these antisense oligos to recurrent and disseminated prostate tumors is currently under investigation.

ACKNOWLEDGMENT

This research was generously supported in part by the Blum-Kovler Foundation and the Cancer Federation.

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